



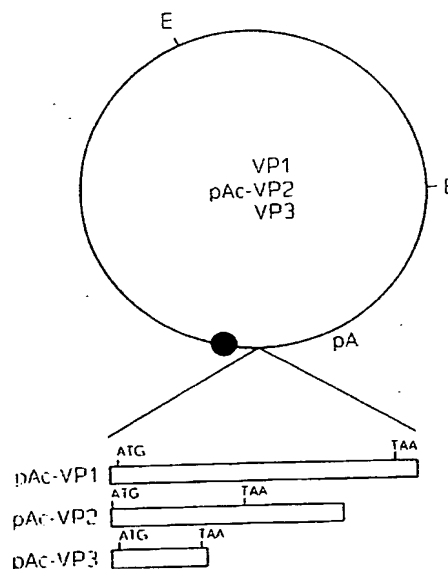
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: CHICKEN ANEMIA VIRUS MUTANTS AND VACCINES AND USES BASED ON THE VIRAL PROTEINS VP1, VP2 AND VP3 OR SEQUENCES OF THAT VIRUS CODING THEREFOR

## (57) Abstract

Novel proteins of the Chicken Anemia Virus are described and compositions for preventing or treating infections with that virus (CAV), in particular vaccines less pathogenic than the CAV itself, but yet leading to neutralizing antibodies in the immunized animal. Besides, there are described compositions containing antibodies against parts of the CAV for the control of infections with CAV and anti-idiotypic antibodies. The invention also provides antibodies and test kits for the detection of CAV. Recombinant DNA molecules derived from CAV and host cells transfected therewith and vaccines based on these host cells are made possible by this invention. The invention also comprises living virus vaccines in which a piece of DNA is brought into a virus infectious to the host. Besides, the invention provides uses of proteins of CAV in the induction of apoptosis, in particular in tumor cells. It further provides the induction of cell death by means of gene therapy.



= polyhedrin promoter  
 ATG = initiation codon  
 TAA = stop codon  
 pA = polyadenylation signal  
 E = EcoRI

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Title: Chicken Anemia Virus mutants and vaccines and uses based on the viral proteins VP1, VP2 and VP3 or sequences of that virus coding therefor.

The present invention relates to novel proteins and/or polypeptides of the Chicken Anemia Virus. Besides, it relates to vaccines and compositions for preventing or treating virus infections in poultry, in particular infections with the  
5 Chicken Anemia Virus (CAV).

In particular, the invention relates to vaccines that are less pathogenic than the CAV itself but yet lead to the generation of neutralizing antibodies in the immunized animal.

Besides, the invention relates to compositions containing  
10 antibodies against parts of the CAV for controlling infections with CAV. Also anti-idiotypic antibodies which possess an immunogenicity corresponding with the antigen are a subject of the invention.

The invention also relates to antibodies for the  
15 detection or control of CAV infections. Also diagnostic test kits for the detection of CAV will be described.

The invention further relates to recombinant DNA molecules derived from CAV, which code for at least an immunogenic part of a CAV protein and host cells transfected  
20 with such recombinant DNA molecules. Vaccines based on these host cells are made possible by this invention.

Also so-called living virus vaccines in which a piece of DNA coding for at least an immunogenic part of a CAV protein is brought into a virus infectious to the desired host are a  
25 subject of the invention.

Processes for the prophylaxis or control of CAV infections, in particular in chickens, and processes for the preparation of recombinant parts of CAV comprising sequences, and processes for the preparation of vaccines are also  
30 subjects of the invention.

Besides, the invention relates to uses of the proteins of the CAV in the induction of apoptosis (programmed cell death). In particular, the proteins (polypeptides) can be used in the induction of apoptosis in tumor cells.

Besides, the proteins according to the invention can also be used in the elimination of other undesired cell populations, such as autoimmune reactive T cells in autoimmune diseases, such as rheumatoid arthritis, lupus, etc.

5       The invention further provides for the induction of cell death by means of gene therapy. Processes for preparing these therapeutics and processes for treatment therewith are also subjects of the invention.

10       The Chicken Anemia Virus (CAV) is a recently characterized DNA virus (Noteborn and De Boer, 1990). It belongs to a new virus family. In young chickens CAV causes anemia by destruction of erythroblastoid precursor cells and immune deficiency by depletion of thymocytes. Lesions occur in the spleen and liver (Jeurissen et al., 1989). A recent study  
15       has shown that the depletion of thymocytes is caused via apoptosis induced by CAV ((Jeurissen et al., 1992b).

      Gelderblom et al. (1989) and Todd et al. (1990) have shown by means of electron microscopic studies that CAV particles have a T3 icosahedron symmetry and a diameter of  
20       23-25 nm. The CAV particles concentrate after equilibrium sedimentation at a density of 1.33-1.34 g/ml in CsCl.

      Todd et al. (1990) have shown that isolated virus particles contain only one protein having a molecular weight of 50 kDa. The single-stranded DNA in the CAV particles is in  
25       the form of a circular minus strand (Gelderblom et al.; Todd et al., 1990; Noteborn et al., 1991). The replicative DNA intermediary was cloned and fully sequenced. The CAV genome is 2319 nucleotides long. On the basis of the genome structure and the DNA sequence the virus cannot be placed into one of  
30       the known virus families (Noteborn et al., 1991; Todd et al., 1991). The CAV genome contains three large, partially or completely overlapping reading frames coding for possible proteins having molecular weights of 51.6, 24.0 and 13.3 kDa. The CAV genome moreover contains one evident promoter/enhancer  
35       region and only one polyadenylation signal. Transcription of the replicative DNA intermediary produces a polyadenylated

polycistronic RNA molecule of approximately 2100 nucleotides (Noteborn et al., 1992b).

Day-old chicks are most susceptible to CAV infections. In these animals lethargy, anorexia and anemia are observed from 5 10 days after inoculation with CAV. After infection mortality may increase to a maximum of 50%. With increasing age the resistance also increases. Jeurissen et al. (1992) have reported that only the hematocrit values of chicks that had been infected with CAV at an age of 1-3 days are decreased. 10 CAV infections of 1-21 days old chicks result in a depletion of in particular the thymus cortex. However, in older chickens CAV can subclinically multiply. CAV infection in older chickens can be determined by the occurrence of serum conversion (McIlroy et al., 1992).

15 The spread of CAV within a flock of chickens substantially occurs via contact infection. Most probable is ingestion of feces or other material contaminated with feces from CAV infected animals. Infection via the air, however, cannot be ruled out. Transmission of viruses to offspring via 20 the egg is suggested by Yuasa et al. (1979) but by way of experiment vertical transmission of CAV from mother animals to chicks could not be demonstrated by us.

Immune deficiency resulting from the CAV induced depletion of the thymus cortex is considered to be the cause 25 of disease symptoms occurring after secondary infections of normally non-pathogenic agents (De Boer et al., 1992; Engström, 1988; Rosenberger and Cloud, 1989; Von Bülow et al., 1986; Yuasa et al., 1980). Thus CAV is isolated in animals with Newcastle disease, Marek's disease, infectious bursitis 30 (Gumboro) and in animals with 'blue wing disease' in association with reoviruses. CAV infections lead to increased inoculation reactions, e.g. against Newcastle disease virus.

Maternal antibodies have been found to give an important protection against CAV infection. A recent study under 35 laboratory conditions has shown that maternal immune day-old chicks develop no CAV infection. Day-old chicks can also be protected passively by intravenous injection of antibodies

from egg yolks of immune mother animals (De Boer et al., dates not published).

CAV can be multiplied in tissue culture. The titers then obtained are low in general. At present MDCC-MSB1 cells  
5 (Yuasa, 1983; Yuasa et al., 1983) are used therefor, in which CAV induces a cytopathogenic effect 48-72 hours after infection. MDCC-MSB1 cells are also used to determine neutralizing antibodies and antibodies directed against CAV by means of immunofluorescence (Von Bülow et al., 1985; Chettle  
10 et al., 1991). It has not been found possible so far to attenuate the virulence of CAV by serial passage in MDCC-MSB1 cells.

Older animals do not develop disease symptoms after CAV infection and chicks with maternal antibodies are protected.  
15 These data were used in Germany in a vaccination program based on controlled exposure to CAV of 14-16 weeks old mother animals. In the Netherlands this vaccination method is not allowed except at experimental level because of the attendant risks. As mentioned above, it is quite possible that CAV can  
20 be transmitted to offspring via the fertilized egg. McNulty et al. (1991) have recently shown that flocks that are CAV seropositive have production numbers inferior to those of CAV seronegative flocks. Moreover, Adair (personal communication) has shown immune deficiency in chickens having a subclinical  
25 CAV infection. The possible vertical virus spread and the immune deficiency caused by CAV with (sub)clinical infections renders a control program based on an innocuous vaccine very desirable.

In general, inactivated vaccines and subunit vaccines are  
30 the safest vaccines. The fact that under tissue culture conditions CAV multiplies only to low titers renders the preparation of an inactivated vaccine relatively expensive and laborious. For the preparation of a subunit vaccine against CAV infections those CAV proteins are necessary which induce a  
35 protective immune response in vaccinated chickens. Thus far only one protein (called VP1) has been found in purified CAV particles.

Surprisingly, it has now been found that this protein alone, as will further be shown in the Examples, is not capable of giving an immune response that protects against CAV infections. It has been found that in spite of the fact that VP1 seems to be the only protein present in the virus particle the VP2 protein now expressed by us for the first time is essential for generating virus neutralizing antibodies. Therefore, it is possible only now to develop an effective vaccine on the basis of parts of the virus.

10 We have cloned the three open reading frames present on the CAV genome into baculovirus vectors. The three CAV proteins VP1, VP2 and VP3 were expressed into Sf9 cells alone, in combination with one of the other CAV proteins or all three simultaneously by means of (co)-infection with recombinant CAV  
15 baculoviruses. Mother animals were injected with crude cell lysates which contained one or more CAV proteins. Only after immunization of chickens with antigen preparations containing proportional amounts of all three CAV proteins or containing essentially VP1 and VP2 and also some VP3, neutralizing  
20 antibodies developed. Eggs of such animals contained maternal antibodies against CAV. Infection tests with offspring of vaccinated mother animals showed that at least the CAV proteins VP1 and VP2 are necessary for the induction of a protective immune response. Offspring of mother animals  
25 injected with all three CAV proteins were even better protected against infections with CAV. Injection into chickens with all three CAV proteins that had each individually been produced in Sf9 cells, induced few neutralizing antibodies against CAV. This implies that for an optimum induction of  
30 neutralizing antibodies against CAV 2 or 3 CAV proteins must be synthesized together in an (insect) cell.

It is possible that fragments of 2 or 3 CAV proteins are already sufficient to effect a protective immune response against CAV infections.

35 The recombinant CAV products, VP1 + VP2 or VP1 + VP2 + VP3, which will be used for vaccination of laying-hens, can be synthesized by means of the baculovirus system. The CAV

proteins can also be synthesized by means of other systems, such as bacterial or yeast cells, via retro(viral) infection or gene amplification (CHO-dhfr system).

5 The fact that 2 or 3 proteins encoded by the open reading frames of the CAV genome can induce a protective immune response in chickens is also applicable to the development of living virus vectors. The coding sequences for VP1 + VP2 or VP1 + VP2 + VP3 are then cloned into living virus vectors.

10 It is also possible that one of the CAV proteins VP1, VP2 or VP3, separately, but then within the context of a living virus vector, is also suitable for the induction of a protective immune response against CAV infections.

The expression of fragments of one or more above-mentioned CAV proteins by living virus vectors may be  
15 sufficient for the induction of a protective immune response.

In poultry, only living virus vectors which themselves show a good replication in the avian system can be used. Eligible for the use of viral vectors in chickens are, among other things: fowl pox virus, retroviral vectors, herpes virus  
20 vectors (Marek's virus and turkey herpes virus), adenoviruses and laryngotrachitis virus. It has been found that the induction of cell death as induced by CAV can essentially be attributed to VP3 and partly to VP2.

By deletion of the C terminal 11 amino acids of VP3 the  
25 induction of apoptosis by VP3 is strongly reduced. Consequently, the pathogenic activity of CAV can be drastically reduced by introduction of a stop codon into the C terminal region of VP3. The extra stop codon in the coding region for VP3 is introduced into the CAV clone pCAV/EcoRI  
30 (Noteborn and De Boer) which contains the complete CAV genome. The complete CAV mutant genome is cut from the vector and recycled. MDCC-MSB1 cells are transfected with the recycled CAV mutant DNA, and the virus offspring which are less pathogenic are harvested. Chickens are vaccinated with the  
35 attenuated CAV mutant viruses. Since the VP2 protein also has an effect on the induction of apoptosis, it is possible to



also prepare attenuated CAV which contains a mutation in the coding region for VP2 or VP2 and VP3.

The above-mentioned introduction of a stop codon into the coding region for VP2 and/or VP3 can also be used in the  
5 production of CAV recombinant living virus vectors.

Animals infected with CAV at an older age develop no clinical symptoms. Yet it seems that such infections may lead to great economic losses for the poultry industry. Immunization of animals with the above-described recombinant  
10 CAV products will lead to an active protection against the above-mentioned subclinical symptoms.

The three CAV proteins which were expressed into the baculovirus system separately or in combination with one or two other CAV proteins can be used for tracing antibodies  
15 directed against CAV. Chickens infected or vaccinated with CAV can thus be traced. One or more CAV proteins can be used in immunoassays, such as 'enzyme-linked immunosorbent assay' (ELISA), immunoperoxidase staining and immunofluorescence assay. For measuring neutralizing antibodies two or more CAV  
20 proteins are required.

Immunization of mice with the 3 CAV recombinant products synthesized in insect cells with CAV recombinant baculoviruses finally produced monoclonal antibodies specific for VP2 and VP3. These monoclonals reacted with specific structures in CAV  
25 infected cells and not with uninfected cells.

By means of the antibodies generated with recombinant CAV proteins, CAV proteins can be traced in organ preparations of CAV-infected chickens. On the basis of these data, reliable diagnostic tests can be developed. The monoclonal and  
30 polyclonal antibodies according to the invention may also be used in other diagnostic assays, suchs as ELISAs, RIAs, SPIAs, immunofluorescence assays and immunoperoxidase staining, optionally together with one or more CAV proteins or fragments thereof.

35 In principle, all known embodiments of immunological diagnostic tests are possible with all available labels, and depending on the test to be carried out and the conditions

under which it must be carried out, a person of ordinary skill in the art will be able to select the most suitable embodiment. Besides, for the purpose of this invention antibodies and/or other proteins/polypeptides are also derivatives and/or fragments, as far as they possess the  
5 desired activity. In the case of antibodies this means that they must at least be able to recognize the antigen.

The antibodies according to the invention may also be used for the passive immunization of poultry. Against the  
10 antibodies according to the invention antibodies can be generated which are a so-called "internal image" of the antigen and can thus be used as such again, in particular in passive immunizations and diagnostics.

CAV induces apoptosis in infected thymocytes. It is  
15 possible that a CAV infection of (human) tumors also results in the cell death of the tumor cells.

In vitro the CAV protein VP3 is in itself capable of inducing apoptosis in chicken mononuclear tumor cells and in diverse human tumor cells.

20 Expression of the CAV protein can therefore also be used for the induction of cell death in (human) tumors. The VP3 protein can be (transiently) expressed in tumors by means of DNA transfection. Expression of VP3 in (tumor) cells may also be take place by infecting the cells with (retro)viral vectors  
25 that contain a coding sequence for VP3. Administration to cells of non-viral components (e.g. liposomes or transferrin-derived vectors) containing VP3 proteins and/or coding sequences for VP3 is a further possibility for the expression/presence of VP3 in (tumor) cells.

30 The above-mentioned uses may also serve for the possible induction of cell death by expression in (tumor) cells of VP2 or VP2 together with VP3.

The CAV proteins VP2 and/or VP3 can be used in treatments for reducing (human) tumor formation. This may take place,  
35 e.g., by injecting the proteins according to the invention directly into a solid tumor or coupling the proteins to a ligand having affinity to a tumor associated antiligand. This

coupling can be effected both chemically and (in case the ligand is also a protein) via making recombinant a fusion protein.

5 The chemical coupling can be effected directly or via a spacer group. Optionally, an inert carrier molecule may be selected, such as an indifferent serum protein, to which both the ligand and the viral protein are attached, whether or not via a spacer group.

10 Examples of frequently proposed combinations of ligand-antiligand interactions are ligand-receptor pairs, such as EGF/receptor, IL-2/receptor, /T cell receptor, antibody/tumor antigen, etc.

15 Preference is to be given to a ligand-antiligand combination that can be internalized by the cell. When a conjugate is selected, it can be advantageous to apply an intrinsic unstable group as a coupling between the viral protein and the ligand, so hat the viral protein in the cell returns in native form. Not in all cases will it be necessary to select an internalizing combination. Tumor cells are  
20 metabolically active and will actively or passively take up substances, i.e. also the proteins according to the invention, via fagocytosis and/or pinocytosis.

The ligand to which the proteins according to the invention can be coupled in any manner whatsoever need not be  
25 a complete ligand. In general, it will be sufficient to use the antiligand binding part. Also derivatives of the ligands in question will be useful as long as they possess the antiligand binding activity. In case the ligand is an antibody, the fact is to be considered that antibodies of  
30 another origin than the type to which they are administered will in most cases lead to an immune response. Besides, this also applies to a number of other protein ligands.

It has meanwhile become sufficiently known that antibodies can be manipulated in such a manner that they  
35 generate no immune response but still recognize the desired antigen.

It will be briefly explained hereinafter how animal antibodies can be made suitable for human use (humanizing), but it may be clear that also adaptations of another type are possible.

5 In the first place, it is possible to chemically remove the constant part from the antibody to be humanized, so as to prepare FAB, FAB'2 or still smaller fragments (Winter et al., 1990). In general, these fragments will at least be less immunogenic. Such fragments can also be prepared by means of  
10 recombinant DNA technology.

Besides, it is possible to replace the constant parts of animal antibodies by their human counterparts by means of recombinant DNA technology (Cabilly et al., 1984; Boss et al., 1984).

15 Besides, it is further possible to inoculate the antigen-binding domains of animal antibodies into antibodies of human origin (Winter et al., 1987).

Known tumor antigens against which antibodies have been generated are, e.g., CEA (carcino embryonic antigen) and the  
20 like.

The invention will be explained in more detail on the basis of the following experimental part. This is only for the purpose of illustration and should not be interpreted as a limitation of the scope of protection.

25

#### EXPERIMENTAL

##### Baculovirus, insect cells and chicken T cells

The recombinant baculovirus pAcRP23-lacZ (Bishop, 1992)  
30 was obtained from Dr. R. Possee, NERC Institute of Virology, Oxford, England, and the genomic DNA was purified as described by Summers and Smith (1987). Spodoptera frugiperda (Sf9) cells were obtained from the American Tissue Culture Collection (no. CRL 1711). Baculovirus stocks were grown in confluent  
35 monolayers and suspension cultures in TC 100 medium (Gibco/BRL) containing 10% fetal calf serum as described by Summers and Smith (1987).

The T cell line MDCC-MSB1 transformed with Marek's disease virus (Yuasa, 1983; Yuasa et al., 1983) was grown in RPMI-1680 medium (Gibco/BRL) containing 10% fetal calf serum; the cells were used for DNA transfection experiments.

5

## EXAMPLE 1

### 1.1 Cloning of CAV DNA

All CAV DNA sequences are originally derived from the plasmid DNA pIc-20H/CAV-EcoRI (Noteborn and De Boer, 1990).  
10 All cloning steps with plasmid DNA were in principle carried out according to the methods described by Maniatis et al. (1982).

The coding sequences of the three CAV proteins VP1, VP2 and VP3 were cloned separately into the baculovirus transfer  
15 vector pAcYM1 (Matsuura et al., 1987), which was obtained from Dr. D.H.L. Bishop, NERC Institute of Virology, Oxford, England. The coding sequence for the CAV protein VP3 and a mutant derived therefrom were cloned into the expression vector pRSV-H20 (Offringa et al.).

20 DNA transformations were carried out in the E. coli strain HB101. All plasmids were multiplied in large cultures under agitation, purified on CsCl gradients, and then by filtration over Sephacryl S-500 columns.

### 25 1.2 DNA transfection

DNA of the recombinant baculovirus AcRP23-lacZ was isolated from extracellular baculoviruses according to a method described by Summers and Smith (1987). The lacZ gene contains a unique cutting site for the restriction enzyme  
30 Bsu361. The AcRP23-lacZ was linearized by digestion with Bsu361. Sf9 cells were transfected with calcium phosphate precipitates of linearized baculovirus AcRP23-lacZ DNA and recombinant transfer vector DNA according to the method of Smith et al. (1983); this is an adaptation of the transfection  
35 protocol of Graham and Van der Eb (1973) for Sf9 cells.

For the transfection of the diverse human and chicken cell lines 10 micrograms of pRSV-VP3, pCMV-VP3, pRSV-tr or

pRSV-tr DNA were resuspended in 25 microliters of Milli-Q water and mixed with 260 microliters of TBS buffer.

15 Microliters of 10 mg/ml DEAE dextran were added to the DNA mixture which was incubated for 30 minutes at room temperature.

The cells were centrifuged at 1500 rpm in a table centrifuge. The medium was replaced by 5 ml TBS buffer, and the cells were carefully resuspended. The cells were pelleted, and the TBS buffer was removed. The cell pellet was carefully resuspended in 300 microliters of DEAE dextran/DNA mix and incubated for 30 minutes at room temperature. 0.5 ml 25% DMSO/TBS were added, and the suspension was incubated for 3 minutes at room temperature. 5 ml TBS were added, and the cells were centrifuged at 1500 rpm in a table centrifuge. The supernatant was removed, and 5 ml tissue medium were added. The cells were resuspended, centrifuged, taken up in 5 ml tissue culture medium and incubated at 37°C-5% CO<sub>2</sub>.

### 1.3 Selection of recombinant CAV baculovirus

The supernatants containing extracellular baculoviruses were analyzed in a plaque assay with neutral red (Brown and Faulkner, 1977) and X-gal (Brown et al., 1991). The lacZ-negative plaques were inoculated on a monolayer of Sf9 cells in microtiter dishes. Five days after infection the supernatants were harvested and stored at 4°C. The cell lysates were analyzed in a dot slot hybridization assay with 32P labeled pIc-20H/CAV-EcoRI DNA as a probe.

Monolayers of Sf9 cells were inoculated with supernatants of cell lysates which strongly hybridized with the labeled CAV DNA probe. Two days after infection the cells were labeled with 3H leucine. The proteins were separated on 14% polyacrylamide (PAA) SDS gels (Laemmli, 1970), made visible by means of a fluorography method and tested for the presence of specific recombinant CAV protein and the absence of the  $\beta$ -galactosidase protein.

### 1.4 Synthesis of crude CAV protein preparations

Recombinant CAV baculoviruses which expressed the expected CAV protein in infected Sf9 cells, were dished up according to the method described by Summers and Smith (1983). Monolayers of Sf9 cells were infected with one type of recombina-  
5 recombina-nt CAV baculovirus having a multiplicity of infection (moi) of approximately 5 plaque-forming units (pfu) per cell. Co-infections of 2 or 3 different CAV recombinant baculoviruses were carried out on Sf9 cell monolayers having a moi of 10 pfu of each recombinant CAV baculovirus per cell.  
10 Three days after infection the infected Sf9 cells were harvested. The crude cell lysates were suspended in PBS buffer.

## EXAMPLE II

### 15 2.1 Immunization of chickens with CAV-specific proteins

Groups of 6 weeks old chicken were injected intraperitoneally and subcutaneously with crude lysates emulsified in complete Freund's adjuvant of  $10^6$  or  $10^8$  Sf9 cells which were infected with one or more recombinant CAV  
20 baculoviruses. As a control a group of 8 animals were injected per immunization experiment with PBS buffer emulsified in complete Freund's adjuvant. On different days after immunization blood was collected, and the serum was analyzed for neutralizing antibodies directed against CAV.

25

### 2.2 Immunization of mother animals against CAV

Four groups of each 16 hens were injected with crude lysates of  $2 \times 10^7$  Sf9 cells, which were simultaneously infected with VP1, VP2, and VP3 recombinant baculoviruses; or  
30 with VP1 and VP2; or with VP1 and VP3; or with VP2 and VP3 recombinant baculoviruses. The cell lysates were emulsified in an equal volume of complete Freund's adjuvant. As a control a group of 16 hens was injected with PBS buffer in complete Freund's adjuvant. Yolk material of eggs of hens injected with  
35 these lysates or with PBS buffer was extracted with chloroform and analyzed for the presence of neutralizing antibodies.

EXAMPLE III3.1 Production and characterization of monoclonal antibodies specifically directed against CAV proteins

5 The monoclonal antibody CVI-CAV-85.1 was obtained by injecting mice intraperitoneally with CAV injected MDCC-MSB1 cells with incomplete Freund's adjuvant. Finally, spleen cells of the immunized mice were fused with P3X63-Ag8.653 myeloma cells (Noteborn et al., 1991).

10 The other monoclonal antibodies directed against CAV antigens were obtained by injecting crude extracts of Sf9 cells infected with the three CAV recombinant baculoviruses into the spleen of 4 BALB/c mice. The sera of the immunized mice were tested for 7 weeks after immunization for neutralizing antibodies against CAV. The spleen cells of the  
15 immunized mice were fused with P3X63-Ag8.653 myeloma cells. Antibodies directed against CAV antigens were tested by different ways: a serum neutralization test; ELISAs based on purified CAV and on crude lysates of Sf9 cells infected with CAV recombinant baculovirus; immunofluorescence tests on CAV  
20 infected MDCC-MSB1 or on Sf9 cells infected with CAV recombinant baculovirus; Western blots of crude lysates of Sf9 cells infected with CAV recombinant baculovirus, and immunoperoxidase staining on thymus coupes of CAV infected chickens.

25

EXAMPLE IV4.1 In vitro neutralization test

The sera of chickens and mice injected with crude Sf9 cell lysates or PBS buffer were diluted 1:2 or 1:4 and then a  
30 two-fold dilution series was made. The diluted sera were incubated for 1 hour with  $10^4$ - $10^5$  TCID<sub>50</sub> CAV-Cux-1 (Von Bülow et al., 1983; Von Bülow, 1985). Approximately one hundred thousand cells of the T cell line MDCC-MSB1 transformed by Marek's disease virus were infected with this mixture of  
35 diluted sera and virus. As controls MDCC-MSB1 cells were infected with CAV which was neutralized with a positive CAV antiserum and a negative serum originating from specific



pathogen free chickens.

#### 4.2 CAV challenge experiments

Fertilized eggs of the five groups of immunized hens were  
5 hatched. The chicks were injected intramuscularly on day 1  
with  $10^{5.5}$  TCID<sub>50</sub> CAV-Cux-1. On 6 and on 14 days after  
infection 5 chickens per group were subjected to section. The  
thymus was analyzed macroscopically and immunohistologically.  
Also, heparin blood was taken, and the blood cells were tested  
10 in a virus reisolation assay. Fourteen days after infection  
heparin blood was collected from all animals to determine the  
hematocrit.

#### EXAMPLE V

##### 15 5.1 Immunohistology and immunofluorescence

Frozen coupes of thymus and bone marrow were made and  
used for immunoperoxidase staining with CAV-specific  
monoclonal antibodies, as described by Jeurissen et al.  
(1988).

20 Cells were fixed with 80% acetone and used for  
immunofluorescence tests with CAV-specific monoclonal  
antibodies and goat anti-mouse IgC conjugated with fluorescein  
isothiocyanate (Niteborn et al, 1990).

##### 25 5.2 Detection of CAV in blood samples

Blood samples of CAV infected chicks were washed thrice  
with PBS and taken up in 1 ml. Twenty microliters of the cell  
suspension obtained were added to  $10^5$  MDCC-MSB1 cells. The  
MDCC-MSB1 cells were 10 times diluted every 4-5 days,  
30 transferred to fresh culture medium, until a CAV-specific  
cytopathogenic effect became visible. If after 10 passages no  
cytopathogenic effect could be observed yet, then the virus  
isolation was considered to be negative. The number of times  
of passage is a measure for the amount of infectious CAV  
35 present in the blood of the infected chicks.

## Results and discussion

### Construction of recombinant CAV transfer vectors

The CAV genome contains three large open reading frames which partially or completely overlap each other. By using  
5 start codons in different reading frames the CAV genome codes for 3 unique proteins. The coding sequences for the CAV proteins were separately (VP1, Fig. 1; VP2, Fig. 2; and VP3, Fig. 3) cloned into the baculovirus transfer vector pAcYM1. Because the VP3 reading frame completely falls within the VP2  
10 reading frame, VP3, in case of expression of VP2, is always synthesized too, though in a clearly lesser degree. The transfer vector pAcYM1 lacks the coding sequences for polyhedrin, the polyhedrin promoter inclusively contains the A-residue of the start codon for the polyhedrin gene and the  
15 3'-non-coding sequences including the polyadenylation signal. On both sides of the polyhedrin sequences are flanking viral sequences. The transfer vector contains prokaryote sequences for multiplication in bacteria (Matsuura et al., 1987).

The plasmid pEP-51.6 (Noteborn et al., 1992a) contains  
20 CAV DNA sequences of positions 791 to 2319. The CAV DNA insertion contains the complete coding region for the protein VP1 flanked by 62 bp 5'- and 117 bp 3'-non-coding DNA sequences. The plasmid pEP-51.6 was partially cut with HindIII, then completely cut with EcoRI, and the 'sticky ends'  
25 were filled by means of Klenow polymerase. A 1.53 kb CAV DNA fragment was isolated. The plasmid pAcYM1 was linearized with BamHI, the sticky ends filled by means of Klenow polymerase and finally treated with alkaline phosphatase (CIP). The 1.53 kb CAV DNA fragment was ligated at the linearized pAcYM1 DNA.  
30 The orientation of VP1 in pAcYM1 DNA was determined by restriction enzyme analysis, and the final construct pAcVP1 is shown in Fig. 4.

Plasmid pEP-24.0 (Noteborn et al., dates not published) contains the 1.15 kb BamHI DNA fragment with CAV DNA sequences  
35 of positions 354 to 1508 (Noteborn and De Boer, 1990). This CAV DNA fragment contains the coding region for VP2 flanked by 26 bp 5'- and 484 bp 3'-non-coding DNA sequences. 106 bp

downstream of the start codon for VP2 the start codon for VP3 is found in another reading frame, and the other coding sequence for VP3. The plasmid pEP-24.0 was treated with BamHI; the 1.15 kb DNA fragment was isolated and ligated at the BamHI  
5 linearized and CIP treated 9.3 kb pAcYM1 plasmid. The final DNA construct pAcVP2 was characterized with restriction enzymes and is shown in Fig. 4.

Plasmid pEP-13.3 (Noteborn et al., dates not published) contains the 0.46 kb BamHI-EcoRI DNA fragment with CAV DNA  
10 sequences of positions 427 to 868 (Noteborn and De Boer, 1990). The CAV DNA fragment contains the coding region for VP3, 58 bp 5'- and 25 bp 3'-non-coding DNA sequences. Plasmid pEP-13.3 was cut with the restriction enzymes BamHI and EcoRI, and a 0.46 kb BamHI-EcoRI fragment was isolated. Transfer  
15 vector pAcYM1 DNA was linearized with BamHI and treated with CIP, and a 9.3 kb fragment was isolated. The two synthetic DNA oligomers 5'-GATCCAACCCGGGTTG-3' and 5'-AATTCAACCCGGGTTG-3' were hybridized to each other and together form a BamHI-EcoRI DNA linker. The DNA linker was ligated at the 0.46 BamHI-  
20 EcoRI, and the 9.3 kb BamHI DNA fragment. The final construct pAc-VP3 was analyzed by restriction enzyme digestions and is shown in Fig. 4.

#### Construction of recombinant CAV baculovirus

25 Each of the three recombinant CAV transfer vectors was transfected separately, together with the recombinant baculovirus AcRP23-lacZ DNA, in Sf9 cells. Transfection occurred with "naked" baculovirus DNA and transfer vector DNA. This baculovirus genome contains, instead of the polyhedrin  
30 gene, the lacZ gen, under the regulation of the polyhedrin promoter. After homologous recombination baculoviruses were obtained which had always incorporated one of the three CAV genes instead of the lacZ gene and thus under regulation of the promoter of the polyhedrin gene. The baculoviruses which  
35 have correctly incorporated the CAV gene no longer contain the lacZ gene. In the first instance, the recombinant CAV viruses were characterized for the absence of  $\beta$ -galactosidase activity

in plaques of baculovirus infected insect cells. Further the integration of CAV DNA sequences in the baculovirus genome was determined by means of a CAV-specific DNA probe in a hybridization experiment.

5

#### Expression of the CAV proteins in Sf9 cells

The expression of the specific CAV proteins in Sf9 cells infected with recombinant CAV was analyzed by protein labeling with <sup>3</sup>H leucine and PAA-SDS gel electrophoresis.

10

The CAV protein VP1 has a calculated molecular weight of 51.6 kDa (Noteborn and De Boer, 1990). Lysates of insect cells infected with recombinant VP1 baculovirus contain a protein of 52 kDa beside baculoviral and cellular products. The 52 kDa protein was absent in lysates of insect cells infected with the baculovirus AcRP23-lacZ and in non-infected cells. In vitro expression of the coding sequence of VP1 resulted in a protein of 52 kDa (Noteborn and De Boer, 1992). Most probably, VP1 is not glycosylated because VP1 which is synthesized in a rabbit reticulocyte lysate and VP1 synthesized in insect cells have the same molecular weight.

20

Translation of the gene coding for VP2, but also containing all coding sequences for VP3, produced in an in vitro system specific CAV proteins of 30 and 28 kDa and a minor amount of a 16 kDa protein product. Translation of only the open reading frame coding for VP3 in an in vitro system, however, produced only a protein of 16 kDa. Expression of VP2 by recombinant VP2 baculovirus in infected insect cells produced specific products of approximately 28 kDa and 30 kDa. Sf9 cells infected with a recombinant-lacZ baculovirus do not contain these CAV-specific proteins. The CAV-specific product of 16 kDa could mostly be demonstrated in very small amounts only. These data show that the recombinant VP2 baculovirus strongly expresses the protein VP2 and expresses VP3 in but a minor degree. A possible explanation thereof is that an internal start codon in a gene lying on the baculovirus genome is used very inefficiently.

30  
35

Recombinant VP3 baculovirus synthesized in infected insect cells a main product of 16 kDa and small amounts of some proteins having molecular weights of approximately 21,000 and 12,000-14,000. In an immunofluorescence assay the CAV-specific monoclonal antibody CVI-CAV-85.1 reacted specifically with Sf9 cells expressing VP3. This monoclonal antibody precipitated specifically only a protein having a molecular weight of 16,000 from lysates of radioactively labeled Sf9 cells infected with VP3 recombinant baculovirus. In a pepscan analysis (Geysen et al., 1984) the epitope of the monoclonal antibody CVI-CAV-85.1 was localized on the N-terminus of VP3. The pepscan analysis is shown in Fig. 5.

Induction of neutralizing antibodies in chickens immunized with recombinant CAV proteins

In case of chicken anemia it has been determined that neutralizing antibodies properly correlate with protection. The CAV protein or several CAV proteins inducing neutralizing antibodies in chickens thus form the basis of a subunit vaccine.

In the first instance we have examined which CAV protein is capable of inducing antibodies against CAV neutralizing in chickens. Groups of 8 chickens at an age of approximately 6 weeks were injected with lysates of  $10^6$  or  $10^8$  recombinant CAV-infected Sf9 cells emulsified in complete Freund's adjuvant. As a control a group of 8 chickens was injected with PBS buffer emulsified in complete Freund's adjuvant. Before the immunization and 2, 4 and 6 weeks after immunization blood samples were taken. None of the control animals injected with PBS in complete Freund's adjuvant developed neutralizing antibodies against CAV (Table 1). Also chickens injected with lysates of  $10^6$  or  $10^8$  insect cells infected with recombinant VP2 or recombinant VP3 baculoviruses developed no neutralizing antibodies against CAV. Of the chickens injected with lysate of  $10^6$  infected recombinant VP1 baculovirus insect cells three chickens, and of the chickens injected with a dosis of  $10^8$

infected cells two chickens developed low titers varying between 1:8 and 1:32.

We conclude that the three recombinant CAV proteins, if injected separately into the chicken, induce no or only very slightly neutralizing antibodies against CAV.

Subsequently, we have studied whether the combination of the three recombinant CAV proteins is capable of inducing neutralizing antibodies in the chicken. To this end, Sf9 cells were infected simultaneously with the three recombinant CAV baculoviruses. Crude lysates of  $10^6$  or  $10^8$  of the infected cells, which therefore contained recombinant VP1 + VP2 + VP3, were prepared. Groups of eight chickens at an age of 6-8 weeks were injected with these lysates emulsified in complete Freund's adjuvant. As a control a group of eight chickens was injected with PBS buffer emulsified in complete Freund's adjuvant. Five weeks after immunization the eight chickens immunized with lysate of  $10^6$  infected cells were all found to have neutralizing titers between 32 and 256, whereas seven of the eight animals immunized with  $10^8$  cells had titers between 16 and 512 (Table 2a). Seven weeks after immunization all the animals of both groups were found to have developed a neutralizing titer against CAV. The group of chickens injected with PBS buffer was found to have developed no demonstrable neutralizing immune response against CAV.

Is it really necessary for the induction of neutralizing antibodies against CAV that the three CAV proteins are synthesized simultaneously in insect cells? To answer this question, Sf9 cells were infected separately with VP1, VP2 and VP3 recombinant baculoviruses. Then the crude cell lysates were combined, mixed with Freund's adjuvant and injected into a group of 8 chickens. As control preparations a crude lysate of Sf9 cells which all synthesized the 3 CAV proteins simultaneously and PBS buffer were used. Both preparations were emulsified in complete Freund's adjuvant and then injected into separate groups of each 8 chickens.

Sera of the group of chickens injected with crude lysates of Sf9 cells in which the 3 CAV proteins were synthesized

separately were found to contain no or only very few neutralizing antibodies against CAV. However, the animals of the control group injected with crude lysates of Sf9 cells which together synthesized the 3 CAV proteins were found, as expected, to have developed a neutralizing immune response. The animals injected with PBS buffer were found to be negative (Table 2b).

#### Neutralizing antibodies in eggs of immunized mother animals

10       The above immunization experiments showed that 3 recombinant CAV proteins expressed together in Sf9 cells induced neutralizing antibodies against CAV. In a next experiment it was examined whether combinations of 2 CAV proteins were also capable of inducing neutralizing  
15 antibodies. Here the antibodies in the yolks of eggs of immunized mother animals were measured.

Four groups of 16 chickens at an age of 33 weeks were injected with crude lysate of Sf9 cells which had been infected simultaneously with different combinations of  
20 recombinant CAV baculoviruses. The preparations containing either VP1 + VP2 + VP3 or VP1 + VP2 induced in most animals neutralizing antibodies clearly demonstrable in their eggs (Table 3). The eggs of chickens injected with preparations containing either VP1 + VP3 or VP2 + VP3 were found to have no  
25 clear neutralizing antibody titer in the yolks. Only the yolks of eggs of one of the examined chickens were found to contain low titers of neutralizing antibodies. The eggs of the control group of 16 chickens injected with PBS buffer were found to contain no neutralizing antibodies.

30       The data from the above-mentioned experiments with recombinant CAV proteins show that VP1 + VP2 together are necessary and sufficient for the induction of neutralizing antibodies against CAV infections. However, a minor amount of VP3 in the VP1 + VP2 preparations cannot be ruled out.

35

#### Protection against CAV challenge in offspring of immunized chickens

Maternal antibodies protect young chicks against clinical symptoms caused by a CAV infection. We have studied which group(s) of chickens immunized with specific recombinant CAV proteins became offspring protected against CAV challenge.

- 5 Here virus was isolated and clinical symptoms characteristic of CAV were observed: atrophy of the thymus, decreased hematocrit and increased mortality.

Groups of between 23 and 35 day-old offspring were challenged with a high dosis of CAV. Six days after infection 10 5 animals subjected to section and having mother animals injected with PBS buffer, were all found to have a macroscopically visibly reduced thymus. In case of offspring of mother animals injected with recombinant VP2 + VP3, 4 of the 5 animals had a small thymus. However, the 5 offspring, 15 subjected to section, of mother animals injected with the 3 recombinant CAV proteins together were all found to have a normal thymus. In the group of offspring of mother animals treated with VP1 + VP2 only 1 of the 5 animals examined was found to have a reduced thymus (Table 4). Fourteen days after 20 infection, again 5 animals per group were subjected to section. All offspring of mother animals immunized with recombinant VP2 + VP3 or PBS buffer suffered from thymus atrophy. The examined offspring of the group of animals injected with the 3 recombinant CAV proteins together were all 25 found to have normal thymuses. Only 1 of the 5 examined chicks of the animals injected with recombinant VP1 + VP2 was found to have a reduced thymus (Table 4). An independent experiment showed that offspring of mother animals injected with recombinant VP1 and VP3 had reduced thymuses, as described for 30 the offspring of mother animals injected with recombinant VP2 and VP3 (Koch, results not published). Fourteen days after infection the hematocrit of all CAV infected offspring was determined. A hematocrit of 27% was selected as the limit for anemia. The offspring of the mother animals injected with PBS 35 buffer were all found to have a strongly reduced hematocrit, with values varying between 7 and 19% (Table 5). Offspring of the mother animals injected with recombinant VP2 + VP3 have a



slightly higher hematocrit on average. In these groups only a single animal had a hematocrit higher than 27. An independent experiment showed that also offspring of mother animals injected with recombinant VP1 and VP3 had a reduced hematocrit (Koch, results not published). Of the 35 examined offspring of the animals injected with preparations containing VP1, VP2 and VP3 only one animal had a deviating hematocrit, whereas in the VP1 + VP2 group 2 of the 29 examined animals had a hematocrit below 27%.

10       The high mortality was observed with offspring of mother animals injected with recombinant VP2 and VP3, 50.9% and with PBS, 48.3%. In the group of offspring of mother animals injected with recombinant VP1 + VP2 + VP3 the mortality is 9% and with VP1 + VP2 15.4%. However, most of the animals died 15 within 5 days after challenge. The mortality caused by a CAV infection is generally somewhat later. For this reason we have distinguished in Table 6 between mortality before day 14 and after day 14 after challenge. The mortality before day 14 is often aspecific, inter alia as a result of injection. The 20 mortality after day 14 is in the group of animals with maternal antibodies against VP1 + VP2 + VP3, 7%; against VP1 + VP2, 0%, VP2 + VP3, 27.4% and in the control group 20.7%. In the VP2 + VP3 group 8 animals died after taking blood samples for determining the hematocrit as a result of the poor 25 condition of the chicks, most probably caused by the anemia. In the PBS group 2 animals died during blood taking. All these animals had a clearly reduced thymus.

      The viremia in the CAV infected offspring was examined by carrying out a virus isolation on blood cells. Heparin blood 30 samples of 5 animals per group were taken on 6 and 14 days after challenge. The offspring of mother animals injected with VP2 + VP3 or PBS, and which had practically no protection against CAV infections, were found to contain relatively high virus titers 6 and 14 days after infection. Six days after 35 infection the offspring of animals injected with VP1 + VP2 + VP3 or VP1 + VP2 were found to contain a clearly lower virus titer than the above-mentioned offspring. Fourteen days after

infection only the group of offspring of animals injected with VP1 + VP2 + VP3 had a clearly lower virus titer than the other 3 groups.

The results of the induction of neutralizing antibodies in mother animals show that the recombinant CAV proteins VP1 and VP2 are very important for the induction of a neutralizing immune response. The infection experiments show that the recombinant CAV protein VP3 gives a supplementary protection in addition to the effect obtained by VP1 + VP2.

10

The production and characterization of monoclonal antibodies against CAV

For the production of monoclonal antibodies against CAV mice were injected with crude lysates of Sf9 cells co-infected with VP1, VP2 and VP3 recombinant baculoviruses. In total, 9 different hybridoma cell lines producing monoclonal antibodies against CAV antigens were obtained.

Western blots with CAV antigens produced with the baculovirus expression system showed that the monoclonal antibodies 111.1, 111.2, 111.4, 112.1, 112.2, 120.1 and 120.2 are strongly directed against VP2 and the monoclonal antibodies 111.3 and 120.3 strongly against VP3. The monoclonal antibodies which strongly react with VP2 all show a weak cross reaction with VP3. Conversely, the monoclonal antibodies directed against VP3 show a weak cross reaction with VP2.

A serum neutralization test showed that none of the monoclonal antibodies obtained had a neutralizing activity against CAV, in spite of the fact that the sera of the immunized mice used for preparing the hybridomas did have a neutralizing activity against CAV.

In a pepscan analysis (Geysen et al., 1984) the epitope of the monoclonal antibody 111.2 was localized in the middle of VP2 (Fig. 6). The monoclonal antibody 111.3 was found to be directed against an epitope at the N terminal end of VP3 (Fig. 7), namely beside the VP3 epitope recognized by the monoclonal antibodies CVI-CAV-85.1 (Fig. 5).

Immunofluorescence showed that monoclonal antibodies directed against VP2 and VP3 recognize specific structures in CAV infected MDCC-MSB1 cells. None of the monoclonal antibodies directed against CAV antigens reacted with uninfected MDCC-MSB1 cells. The VP2-specific monoclonal antibodies recognize other structures than VP3 specific monoclonal antibodies in CAV infected cells.

#### Expression of VP3 in chicken cells induces apoptosis

10 Jeurissen et al. have shown that CAV causes apoptosis in infected thymocytes. We have studied whether 1 of the CAV proteins, in particular VP3, is capable of independently inducing apoptosis in chicken T cells.

The coding sequences for VP3 were cloned into the expression vector pRSV-20H. The 0.46 kb BamHI-EcoRI fragment with CAV DNA sequences of positions 427-868 (Noteborn et al., 1991) were isolated from plasmid pEP-13.3. The CAV DNA fragment contains the coding sequences for VP3, and moreover 58 bp 5' and 25 bp 3'-flanking sequences. The vector pRSV-H20 was linearized with BglII, treated with CIP, and a 4.3 kb fragment was isolated. Two synthetic DNA oligomers 5'-GATCCAACCCGGGTG-3' and 5'-AATTCAACCCGGGTG-3' were hybridized and thus formed a double-stranded BamHI-EcoRI linker. The BamHI-EcoRI DNA linker and the 0.46 kb BamHI-EcoRI DNA fragment were ligated at the 4.3 kb BglII DNA fragment. The final construct pRSV-VP3 contained the coding region for VP3 under regulation of the Rous sarcoma virus promoter and was controlled by restriction enzyme analysis (Fig. 8a; Noteborn et al., 1993).

30 MDCC-MSB1 cells were transfected with DNA of pRSV-VP3 by means of the DEAE dextran method. Fourty-two hours after transfection the cells were fixed and analyzed for VP3 expression by staining with monoclonal CVI-CAV-85.1. The cells were also stained with propidium iodide which very strongly stains DNA of intact nuclei but weakly DNA of apoptotic nuclei (Telford et al., 1992). More than 90% of the transfected cells contained a fine-granular distribution of VP3 in the nucleus

which was stained by propidium iodide. Two days after infection 40% of the cells expressing VP3 were found to contain nuclei which were weakly stained with propidium iodide, and VP3 was present as aggregates. Three days and  
5 later after infection more than 90% of the VP3-containing cells were found to contain VP3 aggregates and DNA which very weakly stained with propidium iodide (Fig. 9). Three days after transfection the DNA of the VP3 transfected cells showed the oligonucleosomal ladder pattern characteristic of  
10 apoptosis.

The VP3 distribution observed in transfected cells fully corresponds with that in CAV-infected MDCC-MSB1 cells. Early after infection (after 1-1.5 day) VP3 is fine-granularly distributed in the nucleus: the cellular DNA is still intact  
15 at this stage. Late in infection (after approximately three days) VP3 forms aggregates in the nucleus (Koch, date not published). The DNA of the CAV-infected cells is fragmented (Jeurissen et al., 1992).

Our conclusion is that VP3 in itself is capable of  
20 inducing the CAV-specific apoptosis in MDCC-MSB1 cells. Expression of pRSV-VP3 DNA coding for VP3 in the monocyte cell line LSCC-HD11 also led to apoptosis in these cells.

Expression of the VP2 protein in MDCC-MSB1 cells also leads to damage to the cellular DNA. Three days after  
25 infection of MDCC-MSB1 cells with DNA coding for VP2, in 20% and after 5 days, in approximately half of the transfected cells the nuclei are weakly stained with propidium iodide. Therefore, also VP2, though in a lesser degree than VP3, seems involved in the induction of the CAV-specific cell death.

30

#### The effect of truncated VP3 on the induction of apoptosis in MDCC-MSB1 cells

VP3 is a protein of 121 amino acids in length, contains two proline-rich pieces, a hydrophobic region and two strongly  
35 positively-charged portions (Fig. 8b). The positively charged regions are possibly nucleus localization signals and/or

DNA-binding domains (Noteborn et al. 1987; Ramakrishnan, 1993).

We have studied whether the basic C terminal end of VP# is involved in the apoptotic activity of VP3. To this end, a truncated VP3 product was made by deletion of 11 codons at the C terminus of the VP3-coding sequences. Plasmid pEP-VP3 was cut with the restriction enzymes BamHI and HindIII, and the 0.38 kb BamHI-HindIII DNA was isolated. Two synthetic DNA oligomers, 5'-AGCTTGATTACCACTACTCCCTGAG-3' and 5'-TCGACTCAGGGAGTAGTGGTAATCA-3', were hybridized and thus formed together the double-stranded HindIII-SalI DNA linker. Plasmid pRSV-H20 was cut with BglII and SalI, treated with alkaline phosphatase, and a 4.3 kb DNA fragment was isolated. The HindIII-SalI DNA linker and the 0.38 kb BamHI-HindIII fragment were ligated in the 4.3 kb BglII-SalI fragment. The final construct pRSV-tr containing the coding sequences for the truncated VP3 protein under the regulation of the RSV promoter (Fig. 8a) was analyzed by means of restriction enzyme and sequence analysis.

MDCC-MSB1 cells were transiently transfected with pRSV-tr DNA and, at different moments after transfection, stained with monoclonal CVI-CAV-85.1 and propidium iodide. Immunofluorescence showed that 42 hours after transfection most of the cells expressing truncated VP3 contained fine-granular VP3 in their nuclei. The cellular DNA was strongly stained with propidium iodide. Three days after transfection still 80% of the cells expressing truncated VP3 had nuclei which strongly stained with propidium iodide (Fig. 9). DNA isolated from MDCC-MSB1 cells on 3 days after transfection with pRSV-tr was found to be much less degraded than DNA isolated from pRSV-VP3-transfected MDCC-MSB1 cells. The fraction of the propidium iodide positive nuclei of cells expressing truncated VP3 slowly declined to approximately 50% on 5 days after transfection. Most of the cells containing truncated VP3 and weakly stained by propidium iodide had a granular VP3 distribution. Only a single cell contained VP3 aggregates.

The expression of truncated VP3 in MDCC-MSB1 cells apparently induces cell death much less efficiently than expression of wild type VP3. It is also remarkable that the VP3 mutant can form much fewer aggregates than wild type VP3.

5

#### Expression of VP3 in human tumor cells induces apoptosis

For the expression of VP3 in human cells the expression vectors pRSV-VP3 (Fig. 8a) and pCMV-VP3 were used. The coding sequences for VP3 were cloned into the expression vector pCMV-neo containing the strong promoter of the cytomegalovirus (CMV) immediate early gene (Boshart et al., 1985). The 0.46 BamHI fragment with CAV DNA sequences of positions 427-868 (Noteborn et al., 1991) were isolated from plasmid pAc-VP3 (Fig. 4). The vector pCMV-neo was linearized with BamHI,  
15 treated with CIP, and a 7.5 kb fragment was isolated. The 0.46 BamHI DNA fragment was ligated at the 7.5 BamHI DNA fragment. The right orientation of the VP3-coding sequence with respect to the CMV promoter in the final construct pCMV-VP3 was determined by means of restriction enzyme analysis (Fig. 10).

20 For the expression of truncated VP3 in human cells the 0.46 kb XhoI-SalI fragment of plasmid pRSV-tr coding for truncated VP3 (Fig. 8a) was provided with blunt ends by treatment with Klenow polymerase and isolated. The vector pCMV-neo was linearized with BamHI, provided with blunt ends  
25 and dephosphorylated by treatment with CIP. The 0.46 kb blunt end DNA fragment was ligated at the 7.5 blunt end DNA fragment. The construct pCMV-tr contains the coding sequences for truncated VP3 under regulation of the CMV promoter (Fig. 10).

30 In the first instance, VP3 was expressed in the 3 human hematopoietic tumor cell lines KG-1, DOHH-2 and K562, and in an immortalized cell line, Jobo-0. The cell lines KG-1 and K562 have been derived from different patients with human myeloid leukemia (Koeffler and Golde, 1980) and DOHH-2 from a  
35 patient with a follicular B-lymphoma (Landegent et al., results not published). Jobo-0 cells were immortalized with the Epstein Barr Virus (Landegent, results not published). The 4

human cell lines were transfected with DNA of pRSV-VP3 (KG-1) or with DNA of pCMV-VP3 (DOHH-2, K562 and Jobo-1). The cells were fixed and analyzed for VP3 expression by staining with monoclonal CVI-CAV-85.1 and induction of apoptosis by staining with propidium iodide. Early after transfection VP3 positive cells were observed with a fine-granular distribution of VP3 in the nucleus which was stained with propidium iodide and VP3 positive cells with nuclei containing VP3 aggregates with nuclei that did not stain with propidium iodide. The percentage of VP3 positive cells with nuclei that did not stain with propidium iodide and contained VP3 aggregates was found for the 4 different hematopoietic cell lines to range between 75 and 95% 5 days after transfection (Fig. 11a). Then K562 cells were transfected with DNA of the plasmid pCMV-tr which expresses C terminal truncated VP3. Expression of truncated VP3 in K562 cells induced the cell death much less efficient than wild type VP3.

Our conclusion is that expression of VP3 in human hematopoietic tumor cells leads to specific induction of apoptosis. Expression of VP3 in the human breast tumor cell line MCF-7 (Lippmann et al. 1980) also resulted in the induction of apoptosis (Noteborn et al., results not published).

In the literature it is described that (human) tumors and tumor cell lines that do not contain functional p53 are less/not susceptible to induction of cell death by chemotherapeutics and radiation treatment (Lowe et al., 1993). The tumor suppressor gene p53 acts as intermediary in the induction of apoptosis by specific anti-tumor agents. We have examined whether VP3 is capable of inducing apopt in human cells that do not possess p53 or possess mutated p53. VP3 was expressed in human osteosarcoma cells by means of DEAE-dextran transfection with plasmid pCMV-VP3. The osteosarcoma-derived Saos-2 cells cannot synthesize p53, and Saos-2/alal43 cells express mutated and thus non-functional p53. As a positive control the U2-OS cell line containing wild type p53 was used (Diller et al., 1990). The results given in Fig. 12a show that

VP3 can induce apoptosis in a comparable degree in cells that are p53<sup>-</sup> (Saos-2 and Saos-2/Ala143) or p53<sup>+</sup> (U2-OS). Six days after transfection most of the VP3 positive cells are apoptotic. Expression of truncated VP3 induced much less efficient apoptosis in Saos-2 cells (Fig. 12b). Our conclusion is that VP3 can specifically induce apoptosis in human tumor cells containing or not containing the tumor suppressor gene p53.

#### 10 Description of the Figures

Figure 1 gives the DNA sequence and the amino acid sequence of the VP1 protein of Chicken Anemia Virus. The numbering of the CAV DNA sequences is as given in Dutch patent no. 9002008.

15 Figure 2 gives the DNA sequence and the amino acid sequence of the VP2 protein of Chicken Anemia Virus. The numbering of the CAV DNA sequences is as given in Dutch patent no. 9002008.

20 Figure 3 gives the DNA sequence and the amino acid sequence of the VP3 protein of Chicken Anemia Virus. The numbering of the CAV DNA sequences is as given in Dutch patent no. 9002008.

Figure 4 shows the diagrammatic representation of the 3 CAV recombinant transfer vectors pAc-VP1, pAc-VP2 and pAc-VP3.

25 Figure 5 shows the pepscan analysis of the monoclonal antibody CVI-CAV-85.1 with peptides (12-mers) derived from VP3. The core sequence PSTVFR, against which the monoclonal CVI-CAV-85.1 is directed, is at positions 12 to 17 of the VP3 amino acid sequence (Noteborn et al., 1991).

30 Figure 6 shows the pepscan analysis of the monoclonal antibody 111.2 with peptides (12-mers) derived from VP2. Monoclonal 111.2 is directed against the epitope GLEDRSTQ which is at positions 109 to 116 of the VP2 amino acid sequence (Noteborn et al., 1991). Only the results obtained with peptides nos. 1 through 140 are shown (extinction of  
35 peptides nos. 141 through 206  $\leq 0.103$ ).



Figure 7 shows the pepscan analysis of the monoclonal antibody 111.3 with peptides (12-mers) derived from VP3. Monoclonal 111.3 is directed against the epitope PTSSR which is at positions 19 to 23 of the VP3 amino acid sequence

5 (Noteborn et al., 1991).

Figure 8. Panel A shows the diagrammatic representation of the 2 expression vectors pRSV-VP3 and pRSV-tr. Panel B shows the amino acid sequence of the CAV protein VP3. The proline residues are printed in italics and the basic amino acids in heavy type. The 11 C terminal amino acids, the codons of which are deleted in the expression vector, are underlined.

Figure 9 shows the kinetics of the apoptotic effect of VP3 or truncated VP3. MDCC-MSB1 cells were transfected with plasmid pRSV-VP3 (o) or pRSV-tr (o), fixed and stained with the monoclonal antibody CVI-CAV-85.1 at different times after transfection. The percentages of the immunofluorescent cells with nuclei which normally stain with propidium iodide are given. Per experiment at least 100 cells were counted which had expressed VP3 or truncated VP3.

Figure 10 shows the diagrammatic representation of the expression vectors pCMV-VP3 and pCMV-tr.

Figure 11 shows the kinetics of the apoptotic effect of VP3 on human hematopoietic (tumor) cells. The cell line KG1 was transfected with plasmid pRSV-VP3, and the cell lines DOHH-2, K562 and Jobo-0 were transfected with plasmid pCMV-VP3. The percentages of the VP3-positive cells with nuclei that weakly stain with propidium iodide, apoptotic cells, are given. Per experiment. Per experiment at least 200 cells were counted.

Figure 12 shows the kinetics of the apoptotic effect of VP3 on human osteosarcoma cell lines. Cells of the cell lines Saos-2, Saos-2/Ala143 and U2-OS were transfected with plasmid pCMV-VP3. The percentages of the VP3-positive cells with nuclei that weakly stain with propidium iodide, apoptotic cells, are given. Per experiment at least 500 cells were counted.

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CLAIMS

1. A polypeptide derived from the Chicken Anemia Virus, free from its natural environment, which polypeptide comprises at least part of one of Figures 1, 2 or 3, and which can induce apoptosis or can either directly or indirectly generate  
5 antibodies against Chicken Anemia Virus.
2. A polypeptide according to claim 1, in which polypeptide a mutation decreasing the capacity of inducing apoptosis is provided.
3. A composition for preventing Chicken Anemia Virus  
10 infection, which comprises at least a polypeptide according to claim 1 or 2 and an adjuvant.
4. A recombinant DNA molecule coding for one or more polypeptides according to claim 1 or 2.
5. A DNA molecule according to claim 4, which comprises at  
15 least part of one of the strands of the nucleic acid sequence of Fig. 1, 2 or 3, or which hybridizes under stringent conditions with a DNA molecule comprising at least part of one of the strands of the nucleic acid sequence of Fig. 1, 2 or 3.
6. A vector, which comprises at least a DNA molecule  
20 according to claim 4 or 5 and conventional regulating elements for expression.
7. A vector according to claim 6, which is of viral origin.
8. A vector according to claim 7, which is of retroviral origin.
- 25 9. A vector according to claim 6, which originates at least partially from Baculovirus.
10. A vector according to claim 6, which originates from a living virus vector.
11. A vector according to any of claims 6-10, which comprises  
30 a second, other DNA molecule according to claim 4 or 5.
12. A vector according to any of claims 6-11, which codes for a chimeric protein.
13. A host cell, which is transfected with a vector according to any of claims 6-12.

14. An insect cell, transfected with a vector according to claim 9.
15. A composition for preventing Chicken Anemia Virus in poultry, which comprises a vector according to claim 10 and an adjuvant.
16. An attenuated Chicken Anemia Virus, which possesses a mutation in its DNA coding for at least one of the viral proteins in such a manner that the capacity of inducing apoptosis is decreased.
17. A virus according to claim 16, in which the mutation is such that the virus does not express the 11 C terminal amino acids of a polypeptide according to Fig. 8b.
18. A composition for preventing Chicken Anemia Virus infection in poultry, which comprises a virus according to claim 16 or 17 and an adjuvant.
19. An antibody or a derivative or a fragment thereof, directed against a polypeptide according to claim 1 or 2.
20. An apparatus for the detection of antibodies against Chicken Anemia Virus, which comprises at least a polypeptide according to claim 1 or 2 and an agent for detection.
21. An apparatus for the detection of polypeptides according to claim 1, which comprises at least an antibody or a fragment or a derivative according to claim 19 and an agent for detection.
22. The use of a polypeptide according to claim 1 in the induction of cell death.
23. The use of a polypeptide according to claim 1 in the treatment of tumors.
24. A process for the treatment of tumors, in which a DNA molecule according to claim 4 or 5 is expressed in a tumor cell.
25. A process according to claim 24, in which the DNA is brought into the tumor cell by a viral vector.
26. A process according to claim 24, in which the DNA is brought into the tumor cell via receptor-mediated uptake.
27. A process according to claim 24, in which the DNA is brought into the tumor cell by means of liposomes.



28. A conjugate for the treatment of tumors, which comprises at least a polypeptide according to claim 1 and a substance having affinity to tumor associated proteins, sugars or lipids.
- 5 29. A conjugate according to claim 28, in which the substance having affinity to tumors is an antibody or a derivative or a fragment thereof.
30. A process according to claim 24, in which the DNA is directly injected into the tumor.
- 10 31. A process according to claim 24, in which the DNA is brought into the tumor cell by means of electroporation.
32. A process according to claim 24, in which the DNA is brought into the tumor cell by means of 'a particle bombardment'.
- 15 33. A process according to claims 28 and 29, in which at least a polypeptide is brought into the tumor cell by means of 'a particle bombardment'.



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M H G N G G Q P A A G G S E S A L S R E G Q P G P S G A A Q G Q V I S N E R S P  
ATGCACGGACCGGCAACCGCGCTGGGGGCACTGAATCGCGCTTAGCCGACAGGGGCACTGGCCCCCAGCGGAGCGGCAAGTAATTTCAAATGAACGCTCTCCA 499

R R Y S T R T I N G V Q A T N K F T A V G N P S L Q R D P D W Y R W N Y N H S I  
AGAAGATACTCACCCGGACCATCAACGGTGTTCAGGCCACCAACAGTTACGGCCGTGGAAACCCCTCACTGCAGAGAGATCCGGATTGGTATCGCTGGAATTACAATCACTCTATC 619

A V W L R E C S R S H A K I C N C G Q F R K H W F Q E C A G L E D R S T Q A S L  
GCTGTGGCTGGCGAATGCTCGGCTCCACGCTAAGATCTGCAACTCGGACAAATTCAGAAAGCACTGGTTTCAAGAATGTGCCGACTTCAGGACCGATCAACCCAAAGCCTCCCTC 739

E E A I L R P L R V Q G K R A K R K L D Y H Y S Q P T P N R K K A Y K T V R W Q  
GAGAAGCGATCTGGACCCCTCCGAGTACAGGTAAAGCGACCTAAAGAAGAAAGCTTGATTACCACTACTCCAGCGCCAGCCCGCAACCCGCAAAAGCGGTATAAGACTGTAAGATGGCAA 859

D E L A D R E A D F T P S E E D G G T T S S D F D E D I N F D I G G D S G I V D  
GACGAGCTCGACAGACCGAGCGCGATTTTACTCCTTCAGAAAGGAGCGGTGGCACCACCTCAAGCGACTTCGACGAAAGATATAAATTTTCGACATCGGAGGAGACAGCGGTATCGTAGAC 979

E L L G R P F T T P A P V R I V \*  
GAGCTTTAGGAAGGCCCTTCACAACCCCCCGGTACGTATAGTGTA 1029

FIG. 2

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M N A L Q E D T P P G P S T V F R P P T S S R P L E T P H C R E I R I G I A G I  
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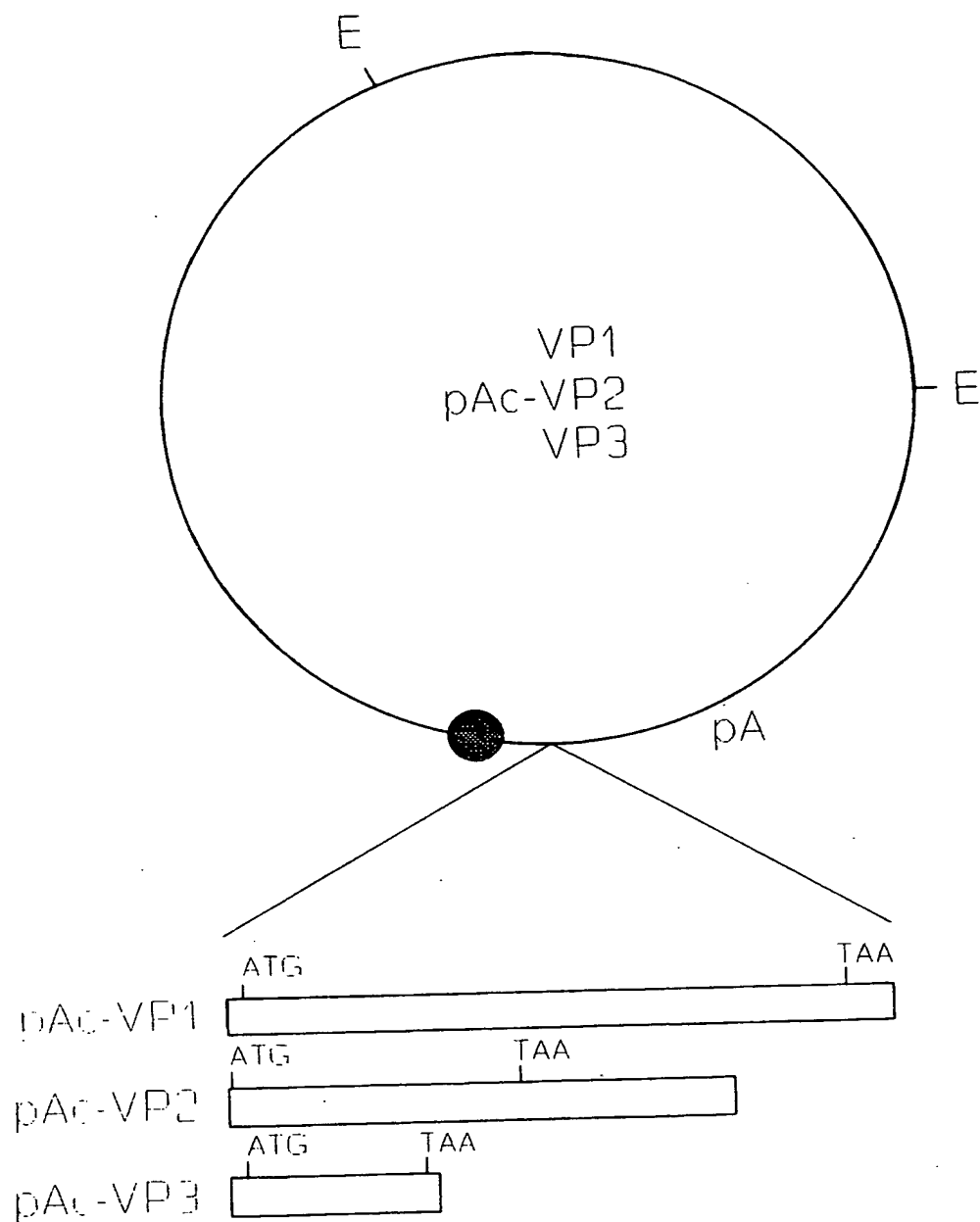
T I T L S L C G C A N A R A P T L R S A T A D N S E S T G F K N V P D L R T D Q  
ACAATCACTCTATCGCTGTGGCTGCCCGAATGCTCCGCTCCACCGCTAAGATCTGCACTCGGACAATTGAGAAACCACTGGTTTCAAGAATGTGCCGACTTCAGGACCGATCAA 725

P K P P S K K R S C D P S E Y R V S E L K E S L I T T P S R P R T A K R R I R  
CCCAAGCTCCCTCGAAGAGCGATCCTGGGACCCCTCCAGTACAGGTAAGCGAGCTAAAGAAAGCTTGATTACCACTACTCCAGCCGACCCCGAACCCGCAAAAAGGCGTATAACA 845

L \*  
CTGTAA 850

FIG.3

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= polyhedrine promotor

ATG = initiatiecodon

TAA = stopcodon

pA = polyadenyleringssignaal

E = EcoRI

FIG. 4

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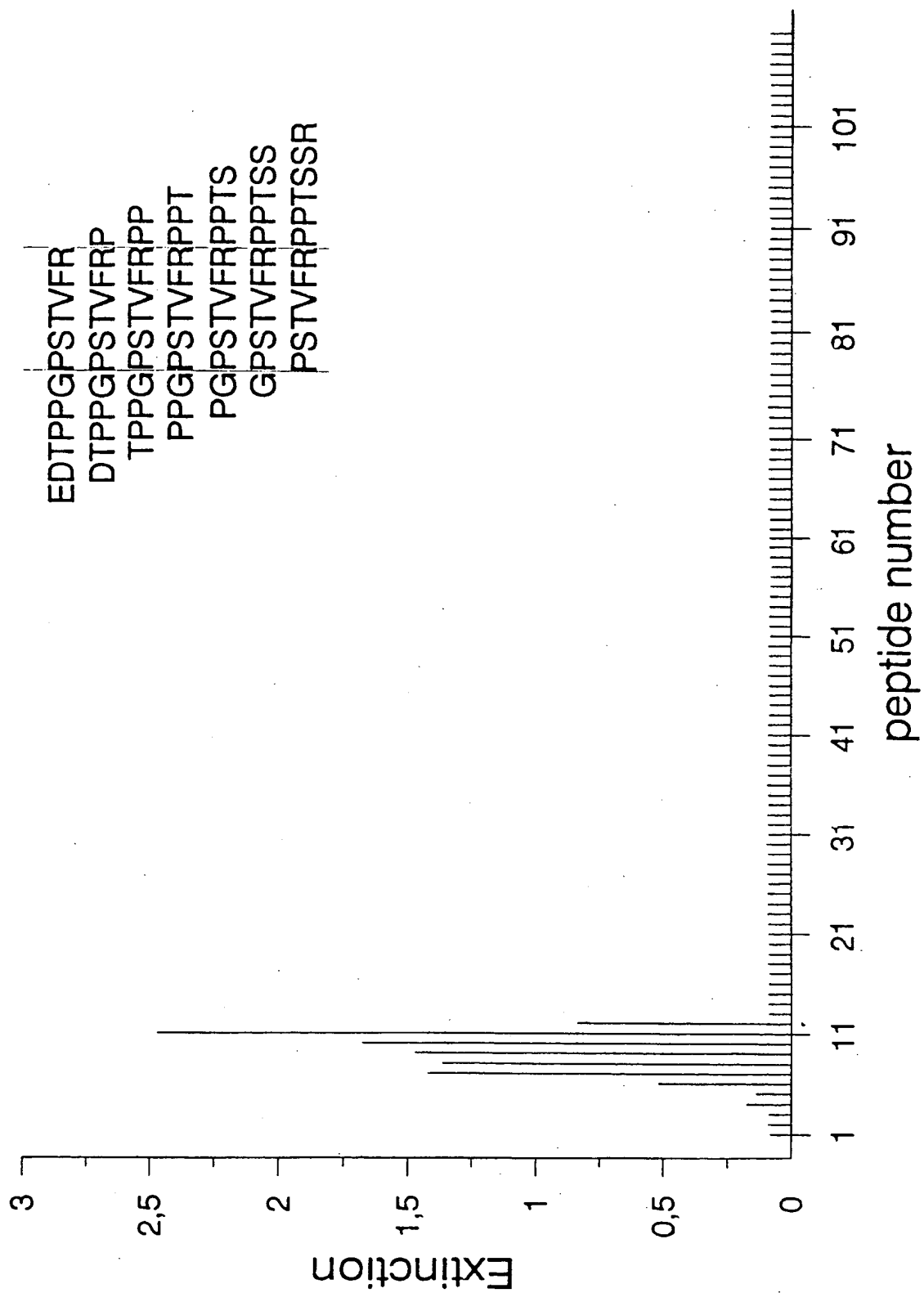


FIG. 5

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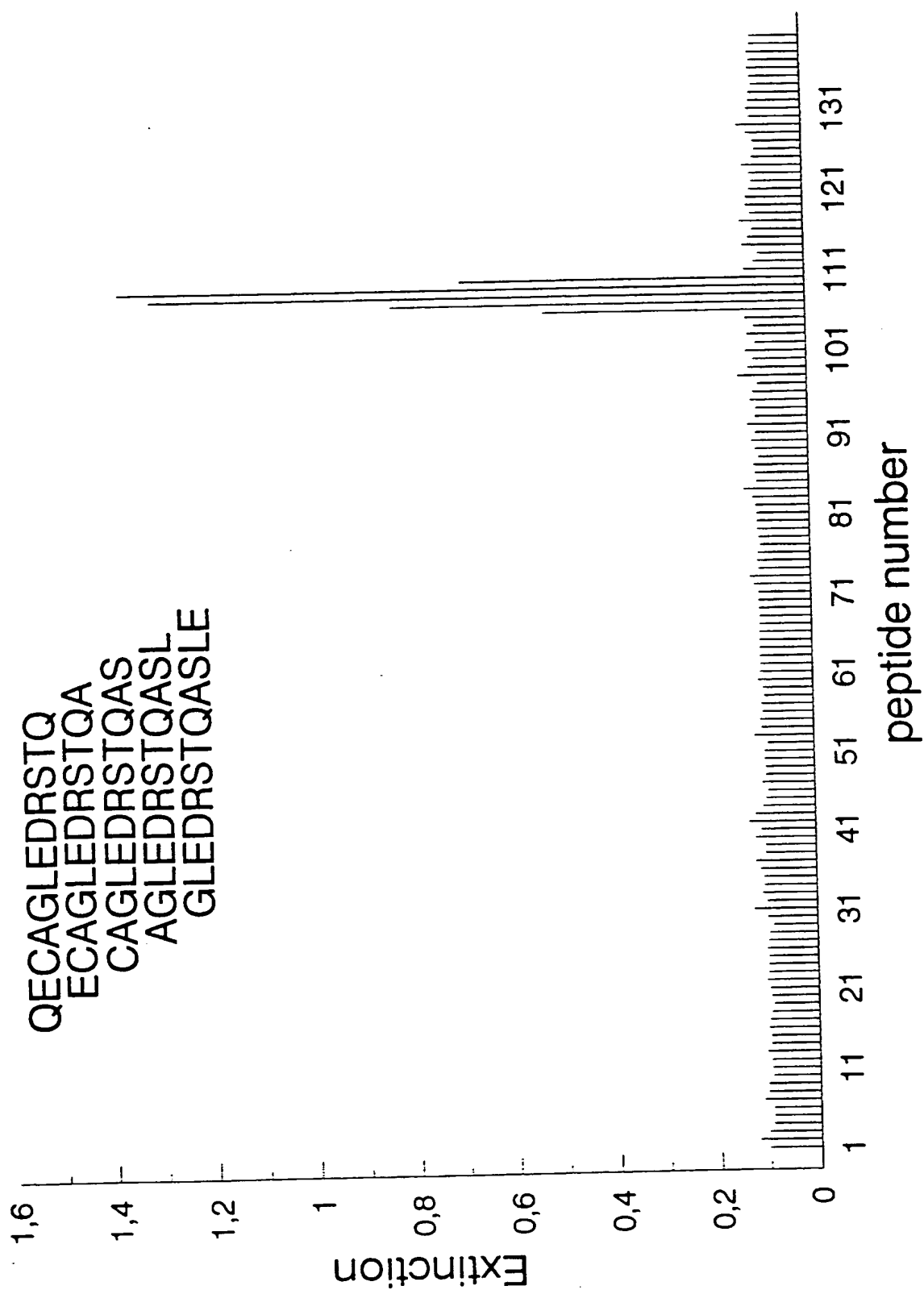


FIG. 6

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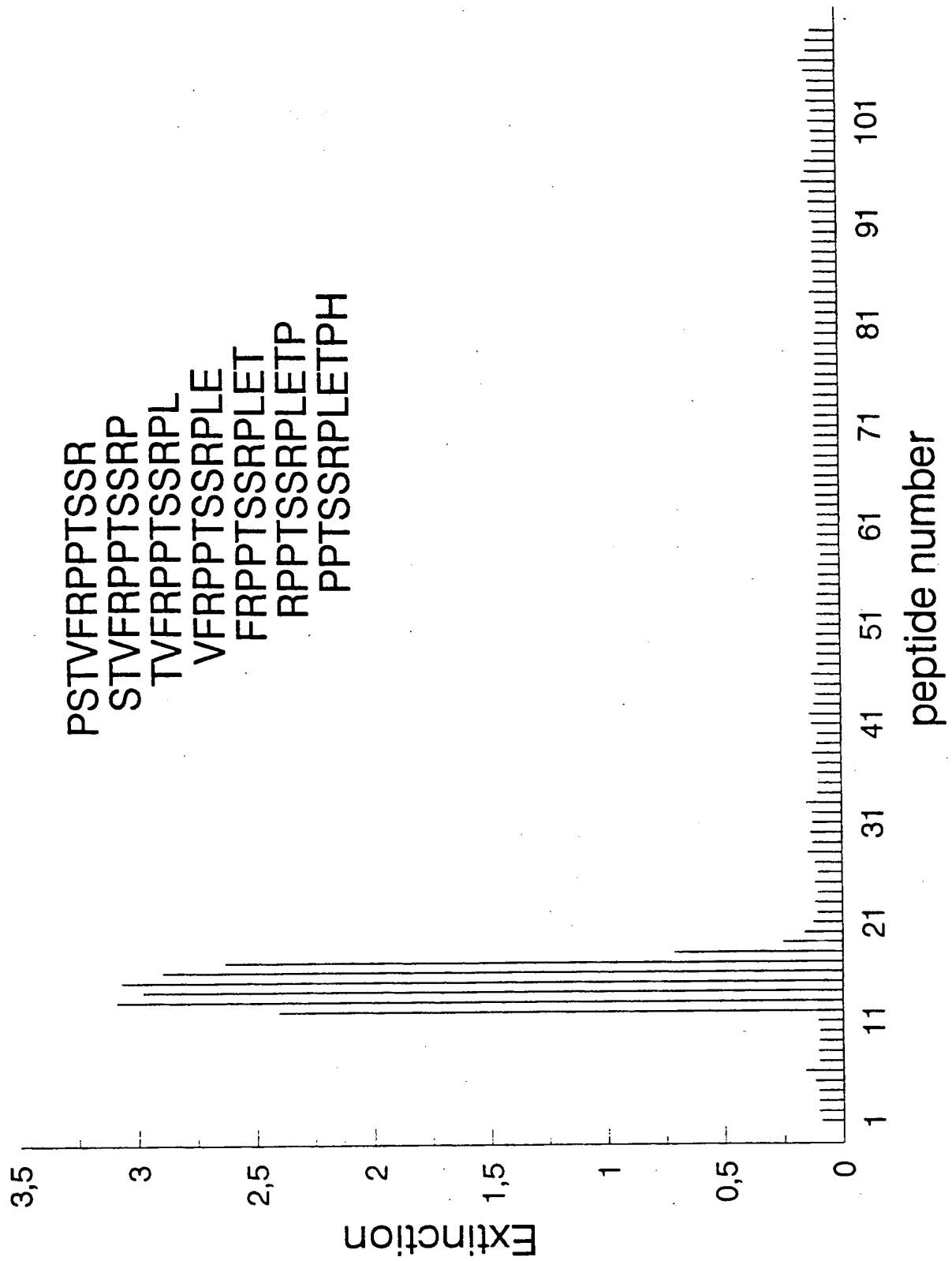


FIG. 7



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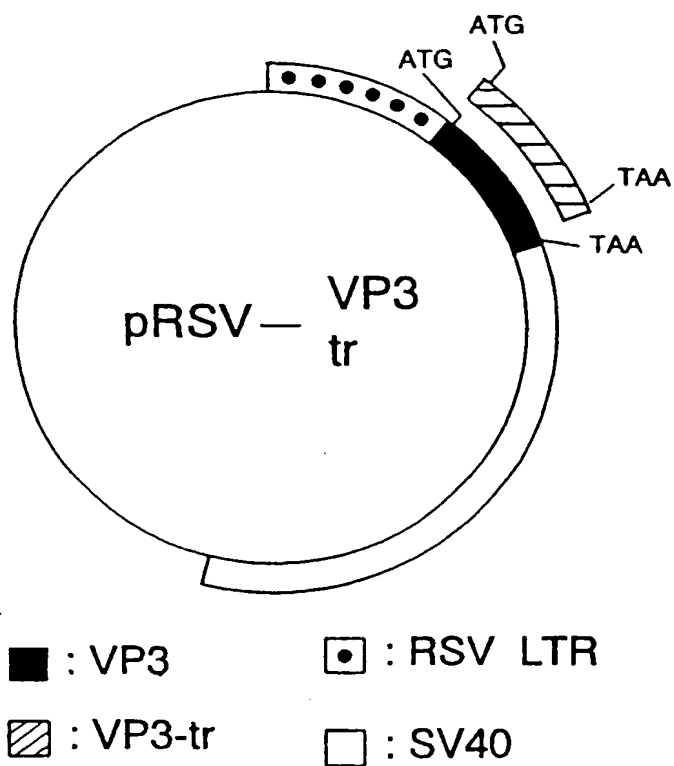


FIG. 8a

## AMINO-ACID SEQUENCE OF VP3.

```

1-M N A L Q E D T P P G P S T V
  F R P P T S S R P L E T P H C
  R E I R I G I A G I T I T L S
  L C G C A N A R A P T L R S A
  T A D N S E S T G F K N V P D
  L R T D Q P K P P S K K R S C
  D P S E Y R V S E L K E S L I
  T T T P S R P R T A K R R I R
  L-121

```

FIG. 8b

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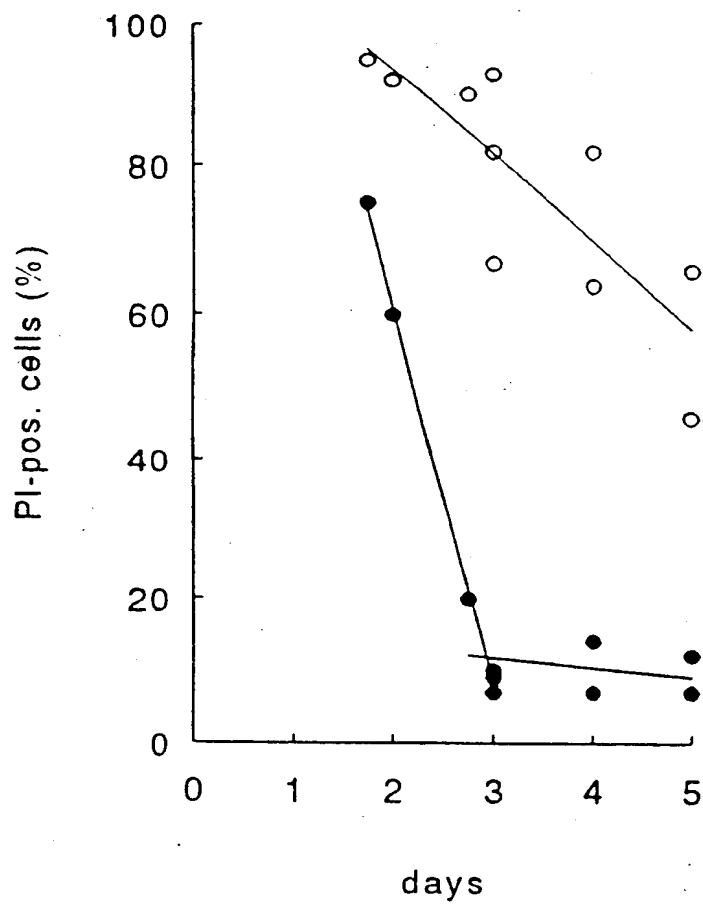
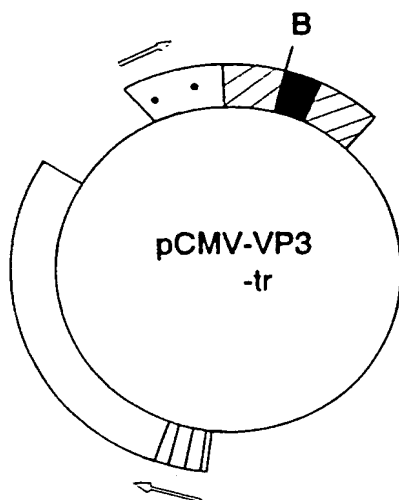







FIG.9

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-  = CMV promoter
-  = rabbit B-globin
-  = neomycin resistance
-  = VP3 or truncated VP3
-  = RSV promoter


-  = pBR322 sequences
- B** = BamHI cloning site

FIG. 10

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## VP3 in human hematologic tumour cell lines

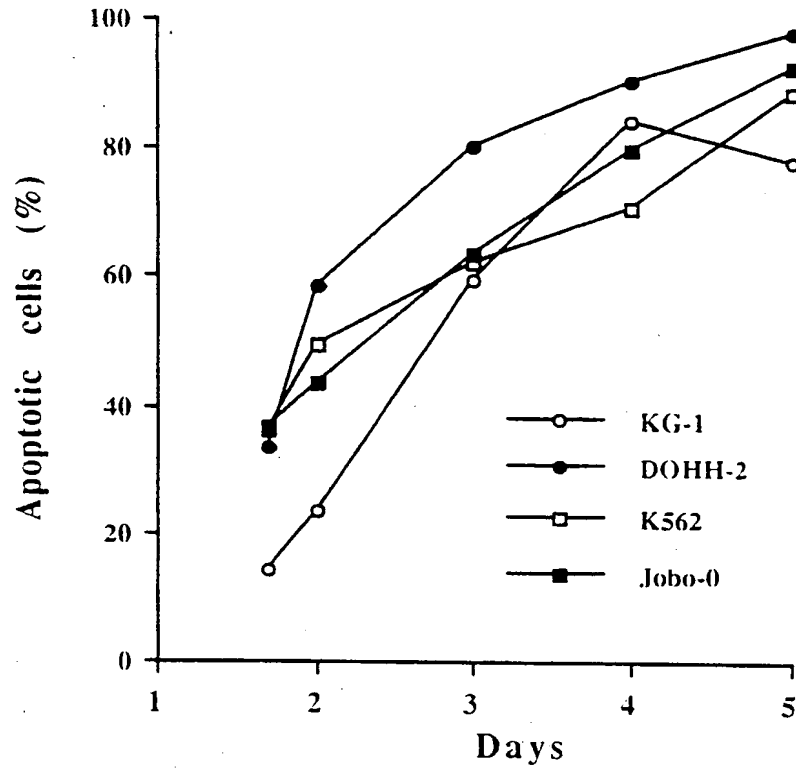


FIG.11a

## Truncated VP3 in K562 cells

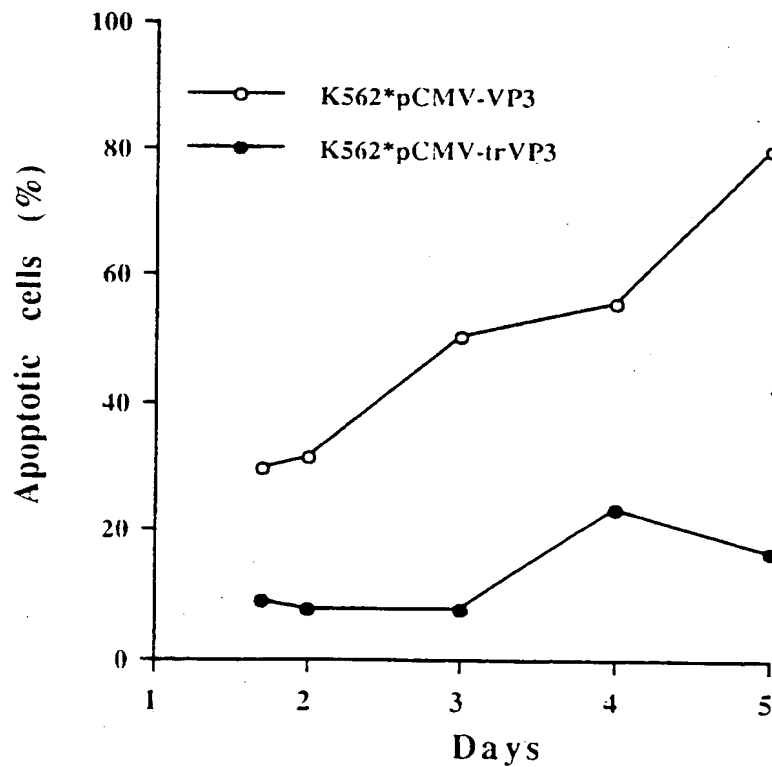


FIG.11b

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## VP3 in human osteosarcoma cell lines

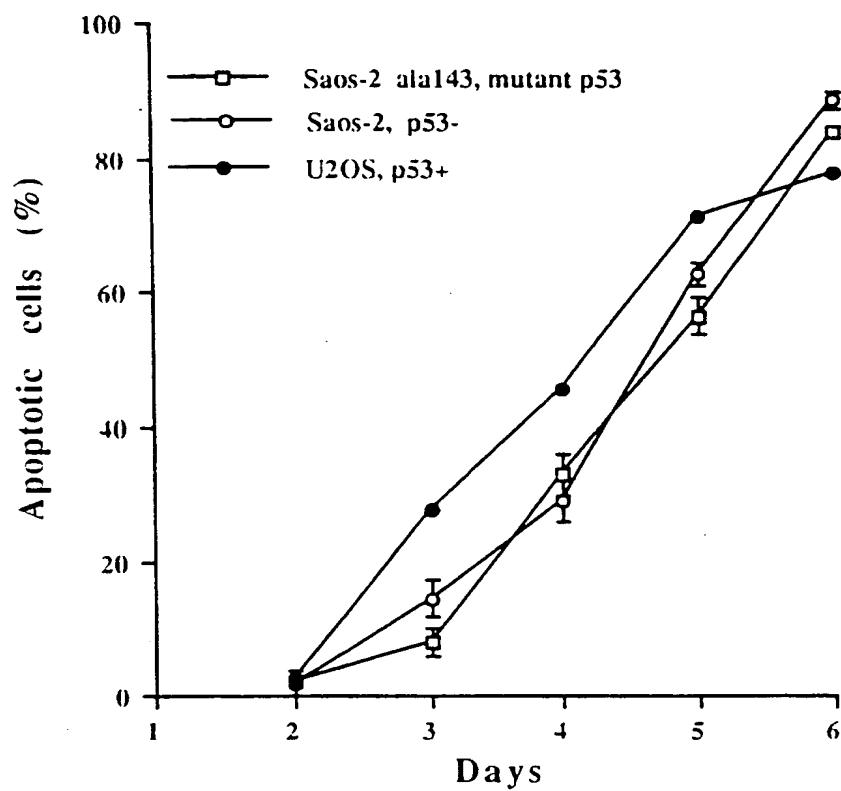


FIG.12a

## Truncated VP3 in Saos-2 cells

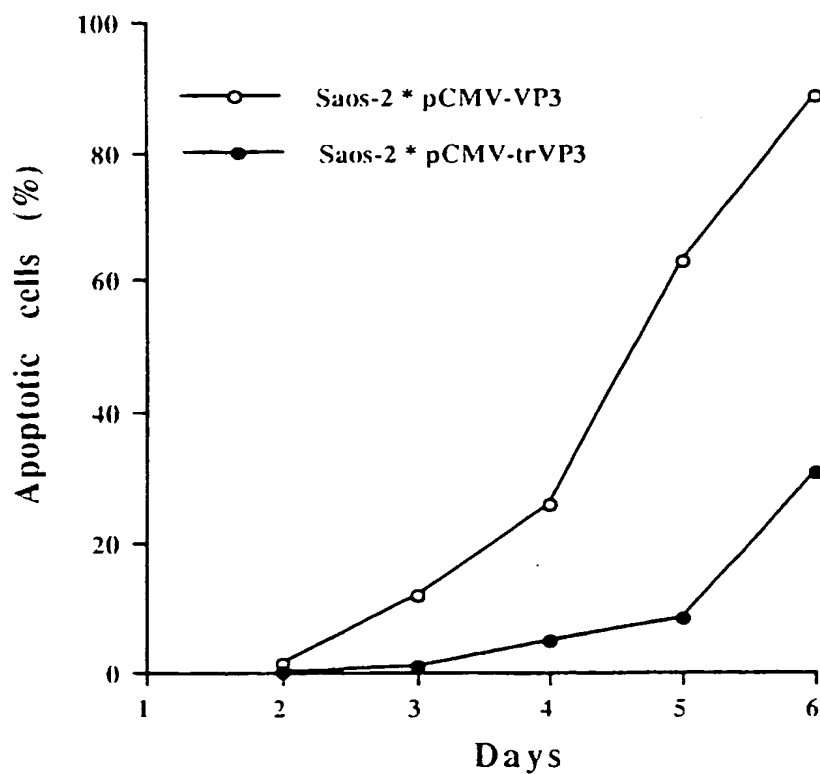


FIG.12b

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